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A Rapid Colorimetric Assay for Sulfur Mustard Cytotoxicity Using Isolated Human Peripheral Blood Lymphocytes and Keratinocytes

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Sulfur mustard (SM) is a potent vesicating agent that has pronounced cytotoxic effects as well as mutagenic, carcinogenic, and radiomimetic properties. Isolated human peripheral blood lymphocytes (PBLs) and human epidermal keratinocytes (HEKs) have been used as in vitro models for determining SM-induced cytotoxicity. A recently developed colorimetric assay (the CellTiter 96 AQ_{ueous} Non-radioactive Cell Proliferation Assay) was assessed using both of the in vitro models described above. Using 24- or 96-well microplates, reproducible (±10%) SM dose/response curves for both types of human cells were obtained using a spectrophotometric microplate reader set at 490 nm. After a 4-h incubation time, as many as 96 sample wells could be measured within 45 s using this commonly available equipment. Multiple plates of samples can be run immediately. This technique may facilitate cytotoxicity investigations of new candidate compounds for both prophylaxis of and therapy for SM intoxication.

Keywords Chromogenic, MTS, Sulfur Mustard, Viability

Sulfur mustard (SM) is a potent blistering agent that has pronounced cytotoxicity (Papirmeister and Davison 1965) as well as mutagenic (Auerbach and Robson 1946) and carcinogenic (Heston 1953) properties. Despite the 8 decades since its first military use in Ypres, Belgium, no effective antidote has yet become available to counteract its ocular, respiratory, and cutaneous effects. The debilitating injuries produced by SM exposure is a continuing problem, but a number of possible avenues of therapeutic intervention have been identified (Smith et al. 1998). Over the past few years, drugs have been synthesized to serve as potential candidates for antivesicant pretreatment and treatment compounds. Some of these drugs have been evaluated using flow cytometry (Meier et al. 1995) to measure cytotoxicity, but this technique is unsuitable for screening processes. A simple, rapid biochemical assay for cytotoxicity that can be performed by laboratory personnel and measured by common laboratory instrumentation would be valuable for increased research productivity.

Swift and convenient methods of measuring the effects of SM on target organisms have relied on in vitro cytotoxicity determinations because in vivo models are complex, costly, and not suitable for large-scale screening efforts. Bacterial and bacteriophage models were first used (Papirmeister 1961) to investigate the biochemical mechanisms responsible for cytotoxicity. The human peripheral blood lymphocyte (PBL) model for SM cytotoxicity was developed (Meier et al. 1987) to answer specific questions about human cells. Human epidermal keratinocytes (HEKs) in culture have also been used to study the mechanisms of SM injury (Smith et al. 1990).

Conventional methods such as the trypan blue assay could be used to measure cytotoxicity caused by SM, but this method is slow and laborious. Flow cytometry using propidium iodide has been a definite improvement in the measuring of cytotoxicity in PBLs and other in vitro systems (Smith et al. 1991) following SM intoxication. This technique is important in determining the effects on basic cellular morphological parameters, such as size, shape, and granularity, and can determine cytotoxicity easily when the proper dyes are used. Some of the shortcomings of this powerful analytical technique are the expensive equipment and the need for specialized and well-trained investigators. This method is also unwieldy when measuring a substantial number of samples.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense of the United States.

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In addition to classical vital dyes such as trypan blue, the thiazolyl blue (MTT) (Mossman 1983) and neutral red (Borenfreund and Puerner 1985) assays utilize the permeability of mitochondrial and lysosomal membranes, respectively, to determine cytotoxicity. However, intermediate procedural steps such as extraction and solubilization limit their usefulness in large investigations. A colorimetric assay, the Promega CellTiter 96 Aqueous (Promega TB #169, Madison, WI), has recently become available; it allows for the quantitation of living cells by measuring the conversion of the unique tetrazolium compound (3-(4,5-dimethyl thiazol-2-yl)-5-(carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) in the presence of an electron coupling reagent, phenazine methosulfate (PMS), by dehydrogenase enzymes in living cells into a yellowish water-soluble formazan. The amount of formazan formed is then directly proportional to the number of living cells in the culture. Moreover, this assay can be used in a desirable tissue culture plate format that requires no additional washing or solubilization steps and can be read immediately in a commonly available spectrophotometric microplate reader set at 490 nm. In addition to circulating cells such as PBLs, this method may also find utility in measuring cytotoxicity in adherent cells in situ without enzymatic detachment and quantification. This assay seemed to be capable of the rapid throughput needed to screen a large number of potential medical countermeasures against SM and was investigated for its potential use in cytotoxicity measurements.

MATERIALS AND METHODS

Reagents

RPMI 1640, trypan blue, gentamicin, and other laboratory chemicals were acquired from Sigma Chemical (St Louis, MO). Percoll was obtained from Pharmacia (Piscataway, NJ). HEKs, keratinocyte growth medium (KGM), and trypsin-EDTA reagent packs were purchased from BioWhitaker (Walkersville, MD). Tissue culture vessels were purchased from Corning (Corning, NY) and Falcon (Newark, NJ). MTS-PMS was purchased in the form of CellTiter 96 Aqueous, Nonradioactive Cell Proliferation kits from Promega. SM (CAS registry #505-60-2, 96.8% pure) was obtained from Soldier and Biological Chemical Command (Aberdeen Proving Ground, MD).

Isolation of Human Peripheral Blood Lymphocytes

Blood was obtained by venipuncture from paid volunteers under an approved human-use protocol. PBLs were isolated as described elsewhere (Meier et al. 1987). Original cell counts and viability determinations were measured by the trypan blue exclusion assay using a Bright Line hemacytometer (Thomas Scientific, Swedesboro, NJ) and following the manufacturer's directions.

Cell Culture

HEKs were purchased as second-passage cells and cultured in KGM in T-75 flasks in a 5% carbon dioxide (CO_2) incubator at 37°C and subcultured into 24-well or 96-well plates. In the 24-well plates, HEKs were usually set up at 12–15,000 cells per well and used about 5 days after inoculation, when confluencies reached 60% to 80%. In the 96-well plates, cells were added at 10 K/well and used about 2 days after inoculation, when confluencies reached 60% to 80%.

Exposure of Lymphocytes to Sulfur Mustard

PBLs were resuspended at 1×10^7 cells/mL in RPMI 1640 and gentamycin (50 μg/mL). A 10-μL aliquot of cells was added to the relevant wells of a 96-well tissue culture plate containing the RPMI buffer. A stock solution of 4 mM SM was diluted into RPMI 1640 in a chemical surety hood and added to the appropriate cell culture plates to yield final SM concentrations varying from 10 to 500 μM (the total volume of each well was 100 μL). The plate was incubated for 1 h at ambient temperature to allow for reaction and hydrolysis of the SM in the approved fume hood and was then transferred to a humidified 5% CO_2 incubator and incubated at 37°C for 24 h.

Exposure of HEKs to Sulfur Mustard

A stock solution of 4 mM SM was diluted into KGM in a chemical surety hood and added to the appropriate cell culture plates to yield final SM concentrations varying from 10 to 500 μM (total volume of each well was 1.0 mL). After 1 h at the ambient temperature to allow for reaction and hydrolysis of the SM, the tissue culture plates were transferred to a humidified 5% CO_2 incubator and incubated at 37°C for 24 h.

Determination of Viability of SM–Exposed Lymphocytes

The 96-well tissue culture plates were removed from the incubator at the appropriate times, and 20 μL of the MTS/PMS solution was added to each well in darkness. The plate was then returned to the incubator and incubated for 4 h, then removed at the end of the incubation period when the absorbance was read on a microplate spectrophotometer at 490 nm. Blank values were subtracted from the data, and viability at each dose was expressed as a percentage of the control viability, as shown:

$$\frac{\text{(Absorbance of sample)}}{\text{(Absorbance of control)}} \times 100.$$

Flow cytometry measurement of viability using propidium iodide was performed as described elsewhere (Smith et al. 1991).

Assessment of HEK Cytotoxicity in 24-Well Plates

Cell viability in 24-well plates was determined using the MTS-PMS assay and read at 490 nm. At 24 h after exposure to SM, the plates were centrifuged at 500g for 10 min at room temperature. A 500-μL portion of the supernatant media was aspirated from each well and discarded. Aliquots of the 50 μL MTS-PMS solution were added to each well, and the plates were incubated at 37°C in a 5% CO_2 incubator for an additional 4 h. Our microplate spectrophotometer could not accommodate 24-well plates, so aliquots of 200 μL supernatant from each well

(in duplicate) were transferred to a clean 96-well plate and read at 490 nm. Blank values were subtracted and viability was calculated as a percentage of control viability using the formula stated above.

Assessment of HEK Cytotoxicity in 96-Well Plates

Cell viability in 96-well plates was also determined using spectrophotometry at 490 nm. After 24 h of SM exposure, the plates were removed from the incubator and 20 μL of the MTS-PMS solution was added. The plates were incubated at 37°C in a 5% CO_2 incubator for an additional 4 h and read at 490 nm using a microplate spectrophotometer. Blank values were subtracted and viability was calculated as percentage of control viability, using the formula stated above.

RESULTS

Various numbers of PBLs (from 1×10^4 to 1×10^6 cells per well) were incubated with MTS-PMS as described in Materials and Methods (Fig. 1). Incubation for longer periods of time did not improve the response. Although there was a linear response to MTS-PMS with low cell concentrations, incubation with larger numbers of cells ($>2.5 \times 10^5$) appeared to saturate the system rapidly. Incubation for 4 h also gave a greater absorbance response than did 2 h.

The number of cells required to provide an optimum linear response was then determined. PBLs were added to give concentrations ranging from 2.5 to 10×10^4 cells per well, and the total volumes were adjusted to either 100 or 150 μL. After adding the appropriate amount of the MTS-PMS reagent (20% of the sample volume), the plates were incubated for 4 h and the absorbance at 490 nm was determined as described. As shown in Figure 2, the assay was linear with both concentrations of PMS-MTS. With the 4-h incubation and 20 μL of MTS-PMS, the slope was 0.057 ± 0.005 and $r^2 = 0.993 \pm .003$ ($n = 3$). Using 30 μL of MTS-PMS, the slope was 0.063 ± 0.003 and $r^2 = 0.998 \pm .007$ ($n = 3$). Similar slopes and less reagent requirement led to the selection of the 4-h incubation and 20 μL of the MTS-PMS reagent.

SM-exposed PBLs were incubated for 24 h and then analyzed for viability by both the MTS-PMS assay and flow cytometry. At low concentrations of SM (10, 50, and 100 μM), the MTS-PMS assay and the flow cytometry technique were significantly different ($p < .05$) based on the paired t-test ($n = 3$) with PBLs from Donor #1 (as shown in Fig. 3), although the dose-response curve appeared similar. Cell viabilities measured by MTS-PMS and flow cytometry at concentrations of 250 and 500 μM SM were identical by t-test analysis ($p > .05$).

At concentrations of SM ranging from 10 μM to 250 μM in PBLs from donor #2, the MTS-PMS assay and the flow

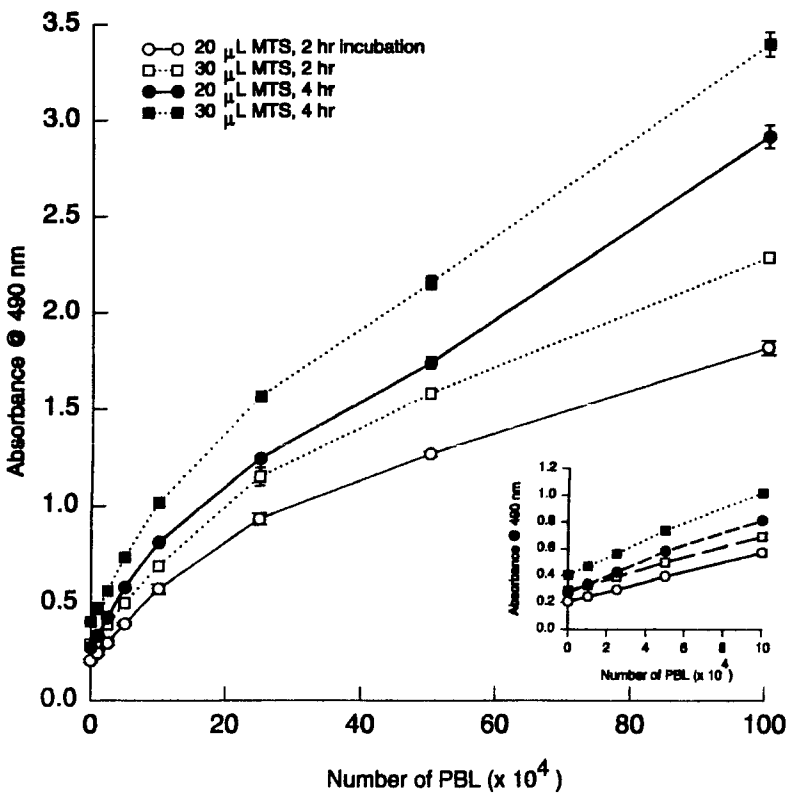

FIG. 1. Effect of cell number and incubation time on the conversion of MTS to the yellowish formazan measured at 490 nm. The MTS-PMS solution was added and the plate was incubated for 2 h and 4 h at 37°C in 5% CO_2. Each point represents the mean ± SEM of six independent observations. The inserted graph portrays the linearity of this assay at lower cell concentrations.

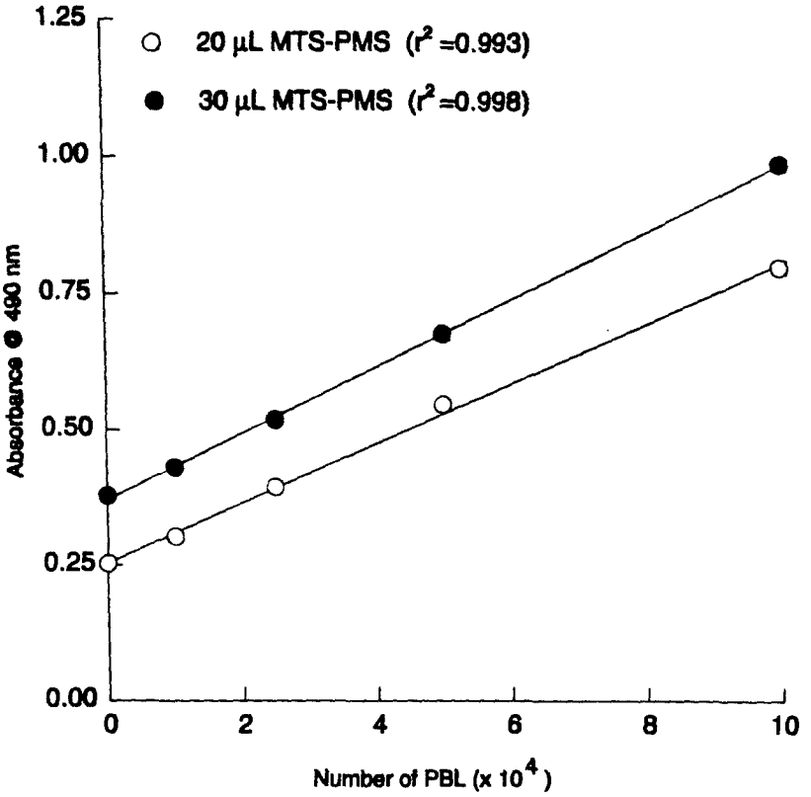

FIG. 2. The dependence of the linearity of the MTS-PMS assay on the number of lymphocytes. Incubation was carried out at 37°C in 5% CO_2 for 4 h, and the absorbance was measured at 490 nm as outlined in the Materials and Methods section. Each point is the mean ± SEM of six separate determinations. Regression values were obtained by Sigma Plot 4.0 software analysis.

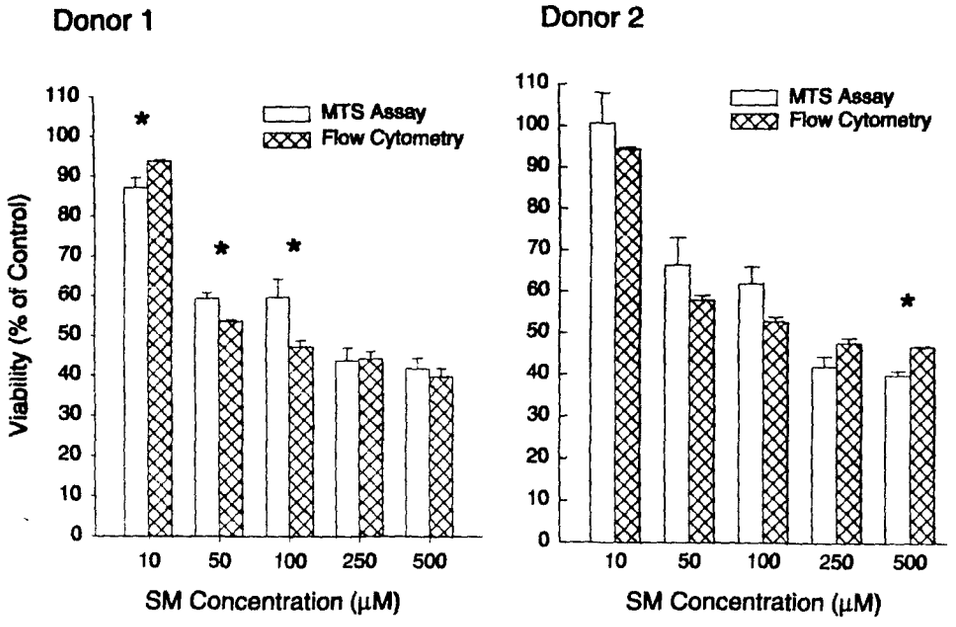

FIG. 3. Comparison of SM cytotoxicity after 24 h in lymphocytes using the MTS-PMS assay and flow cytometry performed as described in the Materials and Methods section. Each bar represents the mean viabilities ± SEM for duplicate experiments. Data were analyzed by the paired *t*-test contained in Sigma Plot 4.0; the level of significance was $p < .05$ (*).

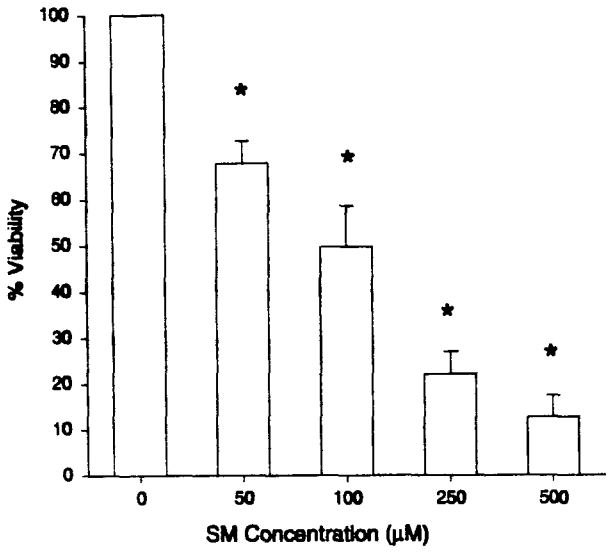

FIG. 4. Determination of SM cytotoxicity in HEK strain #6210 grown in 24-well plates. The MTS-PMS solution was added 24 h after SM exposure and incubated for 4 additional h, as described in the Materials and Methods section. Each bar represents the mean viabilities ± SEM from three separate experiments. Data were analyzed by the paired t-test contained in Sigma Plot 4.0; the level of significance was $p < .001$ (*).

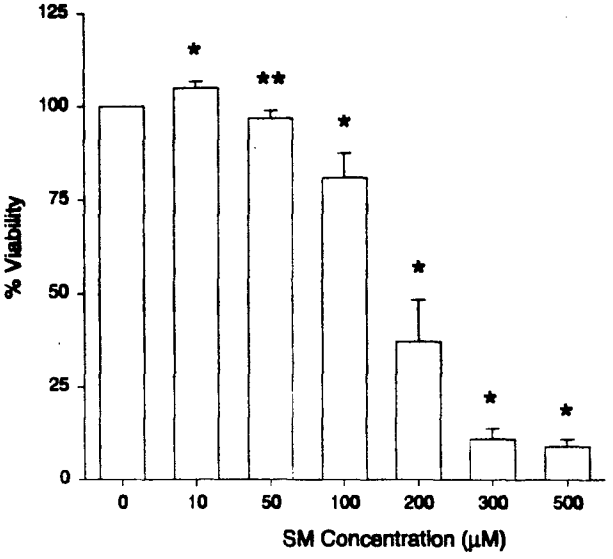

FIG. 5. Determination of SM cytotoxicity in HEK strain #6207 grown in 96-well plates. The MTS-PMS solution was added 24 h, after SM exposure and incubated for 4 additional h as described in the Materials and Methods section. Each bar represents the mean viabilities ± SEM from three separate experiments. Data were analyzed by the paired t-test contained in Sigma Plot 4.0; the levels of significance were $p < .001$ (*) and $p < .05$ (**).

cytometry technique were not significantly different ($p < .05$) based on the paired t-test ($n = 3$). Sensitivity of PBLs to SM varies among individuals (Meier and Johnson 1992) so this result is not unexpected. With donor #2, the agreement between the two methods ended at 500 μM, the maximum concentration studied.

HEKs grown in 24-well plates exhibited an SM-induced cytotoxicity by the MTS-PMS assay (Fig. 4). A conventional trypsin-EDTA treatment to detach these adherent cells for analysis by flow cytometry was therefore avoided and the assay appeared to be quite reproducible. In three separate experiments, viabilities ranged from 67.6 ± 2.0% with 50 μM SM to 12.7 ± 1.9% with 500 μM SM, using HEK strain #6210. Data analysis by the paired t-test showed a significant difference from controls $p > .001$ (*). Between experiments, the variations at each SM concentration were ≤10%. However, the supernatants had to be withdrawn from the 24-well plate and placed in fresh 96-well plates for analysis because our spectrophotometric microplate reader could not analyze 24-well plates.

HEKs grown in 96-well plates exhibited an SM-induced cytotoxicity by the MTS-PMS assay (Fig. 5). These adherent cells did not have to be transferred to new plates for the measurement of absorbance. Using HEK strain #6207 in three separate experiments, the assay appeared to be quite reproducible, showing a viability of 96.8 ± 2.1% at 50 μM SM and 9.0 ± 1.9% at 500 μM SM. Data were analyzed by the paired t-test contained in Sigma Plot 4.0, and the levels of significance were $p < .001$ (*) and $p < .05$ (**). Different strains of HEKs appear also to have different sensitivities to SM, as was shown before with PBLs from different individuals (Meier and Johnson 1992). The plate containing 96 samples could be read within 45 s, and subsequent plates could be read immediately.

CONCLUSIONS

The Promega CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay can be used conveniently to ascertain SM cytotoxicity using PBLs or HEKs in a 96-well tissue culture plate. The assay requires one simple addition of the MTS-PMS substrate to cell suspensions and can be read by a microplate reader at 490 nm after a 4-h incubation. Cell harvesting procedures and lengthy washing steps are unnecessary. Unlike the commonly used MTT, which requires extraction of the insoluble purple formazan from cells and subsequent measurement at 570 nm, the formazan that is formed in the MTS-PMS assay is water-soluble, and no solubilization steps using volatile organic solvents are required. The plates can be read directly after incubation and then returned to the incubator for further color development if necessary. According to the product literature, the assay can also be stopped by the addition of 10% sodium dodecyl sulfate to each well. The plate is then transferred to a humidified atmosphere protected from the light, and the absorbance can then be measured within 18 h.

The optimum number of PBLs needed to perform the SM cytotoxicity studies is 1×10^5 cells per well instead of the higher concentration of lymphocytes needed for other methods. A number of studies can be run at the same time because an entire plate can be read within 45 s at 490 nm, which is the absorbance peak for the formazan. The only equipment needed for this assay is a spectrophotometric microplate reader with the appropriate filters.

Because this assay worked with human peripheral cells, it was of interest to determine the feasibility of using it for adherent cells. Previous methods to determine the cytotoxicity in HEKs required a lengthy trypsin-EDTA treatment to detach the cells.

The cells could be dissociated into single-cell suspensions for trypan blue determinations or for flow cytometry analysis using propidium iodide or other vital dyes. Both methods are laborious and cannot be easily adapted for screening compounds swiftly. In addition to the ability to screen samples rapidly, the MTS-PMS assay also obviates the necessity for enzyme-mediated steps to detach adherent cells such as HEKs and eliminates the unknown effects of this detachment on potentially moribund cells. The in situ nature of this assay offers a strong advantage over previously employed cytotoxicity methods. The MTS-PMS assay may substitute for flow cytometry analysis in the assessment of drugs that reduce cytotoxicity in PBLs or HEKs after exposure to SM.

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